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NICOTINIC CHOLINERGIC RECEPTORS IN RAT BRAIN

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We have examined the molecular bond requirements of [^{3}H]acetylcholine ([^{3}H]ACh) nicotinic binding sites in rat brain. Reduction of disulfide bonds in vitro with the reducing agent dithiothreitol (DTT) resulted in a decrease in the number of [^{3}H]ACh binding sites that could be measured, but the affinity of the remaining sites was unaltered. The effect of DTT was concentration-dependent, and it was reversed by reoxidation of the reduced disulfide bonds with 5,5-dithiobis-2-nitrobenzoic acid. The reversibility of the DTT effect was prevented by p-chloromercuribenzoic acid, which forms thiol complexes with exposed sulfhydryl groups. The data indicate that disulfide bonds at or near the [^{3}H]ACh recognition site are critical for binding of acetylcholine.

We have also studied the in vivo regulation of nicotinic [^{3}H]ACh recognition sites in brain. Repeated injections of nicotine for 5-21 days increase the number of [^{3}H]ACh recognition sites in cerebral cortex, striatum, thalamus, and hypothalamus. Conversely, chronic inhibition of cholinesterase enzymes with diisopropyl fluorophosphate (DFP)

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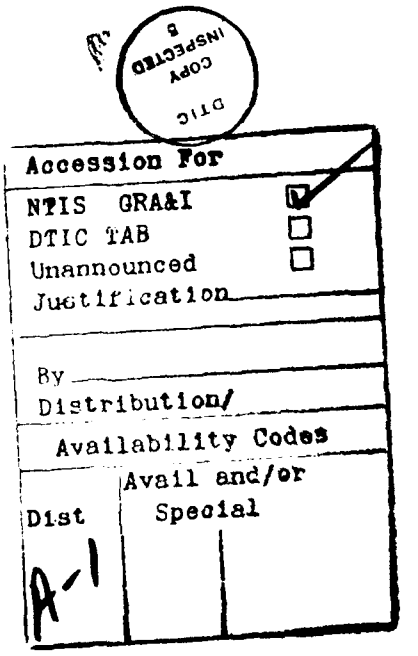
19. (Abstract continued)

decrease the number of [^{3}H]ACh recognition sites. The data indicate that [^{3}H]ACh recognition sites are subject to up and down regulation. The down-regulation of [^{3}H]ACh sites following chronic cholinesterase inhibition indicates that the sites are responsive to synaptic concentrations of acetylcholine. The up-regulation of sites following repeated nicotine administration suggests that nicotine induces a state of prolonged desensitization at the recognition site.



Foreword

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

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During the last year, we have conducted studies to determine:

(1) Whether [^{3}H]acetylcholine ([^{3}H]ACh) binding sites in rat brain, which we hypothesized are recognition sites on nicotinic cholinergic receptors, require disulfide bonds.

(2) Whether [^{3}H]ACh binding sites in brain are affected by repeated administration of nicotine or chronic cholinesterase inhibition.

(3) Whether [^{3}H]ACh can be used to map the brain distribution of nicotinic cholinergic recognition sites using autoradiographic methods.

This report describes the results of those studies.

1. Disulfide bond requirement of [^{3}H]ACh recognition sites in brain. In electroplax and in skeletal muscle, modifications of disulfide bonds decrease the potency of agonists at nicotinic cholinergic receptors (1-3). Modification of disulfide bonds has also been reported to decrease transmission in frog sympathetic ganglia (4); and Karlin (5) has concluded that a reducible disulfide bond at or near acetylcholine recognition sites may be a common feature of nicotinic cholinergic receptors in a variety of peripheral tissues. We have used [^{3}H]ACh to label high affinity nicotinic cholinergic recognition sites in rat brain and to determine whether binding of the agonist is affected by modification of disulfide bonds.

Cerebral cortex from male Sprague-Dawley rats (250-300 g; 3 months of age) was homogenized in 50 mM Tris-HCl buffer (pH 8.5) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1.5 uM atropine sulfate. The homogenates were washed once by centrifugation and then preincubated in dithiothreitol (DTT) to reduce disulfide bonds, with p-chloromercuribenzoic acid (PCMB) to form stable thiol complexes with sulfhydryl groups, or with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to reoxidize sulfhydryl

groups. In each case the tissues were preincubated with the modifying agent for 30 min at 0°C. Each preincubation with modifying agent was terminated by dilution with cold buffer, centrifugation at 48,000 g, and resuspension in fresh buffer with or without another modifying agent. Finally, the tissue was washed twice by centrifugation in fresh buffer containing no modifying agents and then used for measurement of [^{3}H]ACh binding sites as previously described (6). In all cases, control homogenates were carried through the same number of preincubation and washing steps. Differences between groups were analyzed statistically by Student's t-test or Duncan's new multiple range test (7).

Preincubation of rat cerebral cortex with DTT decreased specific binding of [^{3}H]ACh. The IC_{50} of DTT for decreasing binding was approximately 500 uM. Analyses of saturation binding experiments by Scatchard plots indicated that the decreased binding was due to an apparent decrease in the number (B_{max}) of [^{3}H]ACh recognition sites and that the affinity (K_d) of the remaining sites was unaltered. An example of this decrease in [^{3}H]ACh binding sites with increasing concentrations of DTT is shown in Figure 1.

The most prominent effect of DTT is reduction of disulfide bonds to sulfhydryl groups. If the presence of disulfide bonds is critical to binding of [^{3}H]ACh, then reoxidation of sulfyhdryl groups should restore binding. Preincubation of the tissues with the oxidizing agent DTNB alone had no effect on [^{3}H]ACh binding (Table 1). However, the decrease in binding by preincubation with DTT could be reversed by a second preincubation with 1 mM DTNB (Table 1). This effect was due to restoration of the number of binding sites. However, if the sulfydryl groups were reacted with PCMB to form stable thiol complexes

before preincubation with DTNB, the restoration of binding sites by DTNB was at least partially prevented (Table 1).

Scatchard analyses indicated that the apparent affinity of nicotinic cholinergic recognition sites for [^{3}H]ACh was not affected by reduction of disulfide bonds with DTT (Fig. 1). The effects of disulfide bond reduction on other agonist and antagonist affinities were also studied by comparing competition curves in control and DTT-treated membranes. The affinities of neither the agonist cytisine nor the antagonists d-tubocurare, hexamethonium, and dihydro-ß- erythroidine were significantly altered by DTT (Table 2).

To determine whether agonist occupation of the [^{3}H]ACh recognition sites could protect the disulfide bond from cleavage by DTT, preincubation with DTT was carried out in the presence of agonists. The presence of 100 uM ACh or (-)nicotine before and during the preincubation with DTT failed to prevent the reduction of [^{3}H]ACh binding (Table 3).

The results of these studies indicate that high affinity nicotinic cholinergic recognition sites in mammalian brain share with peripheral nicotinic cholinergic receptors the property of dependence on intact disulfide bonds. The inability of agonists to prevent the decrease in binding by DTT suggests that the critical disulfide bond(s) is not directly at the recognition site, but is close enough that when reduced to sulfhydryl groups it renders the site incapable of binding [^{3}H]ACh. It is possible that breaking the disulfide bond causes a conformational change in the receptor so that agonists are no longer recognized. The modification of these [^{3}H]ACh binding sites by alternation of disulfide bonds and sulfhydryl groups should be a useful tool for determining the function of the brain [^{3}H]ACh recognition sites.

2. In vivo regulation of [^{3}H]ACh recognition sites by nicotine and cholinesterase inhibition. An important characteristic of most neurotransmitter receptors is that they are capable of undergoing increases and decreases in number (density) in response to *in vivo* treatments that decrease and increase their stimulation, respectively. This up-and-down-regulation appears to be an important mechanism for regulation of neurotransmission signals, and it is one indication that a binding site is part of a functionally important receptor.

Effects of cholinesterase inhibition. To examine the *in vivo* regulation of [^{3}H]ACh nicotinic recognition sites in brain, the effects of chronic cholinesterase inhibition by diisopropylfluorophosphate (DFP) and the effects of repeated administration of nicotinic agonists on [^{3}H]ACh recognition sites were measured. Rats were injected subcutaneously once daily for 10 days with DFP (1 mg/kg on day 1, and 0.2-0.4 mg/kg on days 2-10). Control rats received injections of water on the same schedule. The rats were killed by decapitation 24 hr after the last injection. Nicotine (1 mg/kg = 6 umol/kg), cytisine (6 umol/kg), or cotinine (6 umol/kg) was injected subcutaneously twice daily for 1-21 days. Control rats received injections of water. The rats were usually killed 18 hr after the last injection, but some rats were killed 1 hr after a single injection of nicotine, and some rats were killed 7 days after a 10-day treatment with nicotine. In some experiments, the effects of the nicotinic antagonists mecamylamine and dihydro-ß-erythroidine alone or preceding the nicotine injections were examined.

Nicotinic cholinergic receptor recognition sites in cerebral cortex, thalamus, striatum, and hypothalamus were assayed using [^{3}H]ACh as previously described (6). Cholinesterase enzyme activity was measured according to the method of Ellman et al. (8). Differences

between or among groups were compared statistically by Student's t-test or by Duncan's new multiple range test when more than two groups were compared.

Cholinesterase enzyme activity in brain was inhibited by 82 ± 2 percent 24 hr after the last of the 10-day treatments with DFP. [^{3}H]ACh binding in cortex, thalamus, striatum and hypothalamus was decreased by 20-38 percent by treatment with DFP (Table 4). The decrease in binding was due to a reduction in the number of [^{3}H]ACh recognition sites, while the affinity of the sites was unchanged (Fig. 2).

This decrease in [^{3}H]ACh recognition sites following chronic cholinesterase inhibition appears to be an *in vivo* adaptive response to the increased synaptic levels of acetylcholine. This indicates that [^{3}H]ACh recognition sites are responsive to synaptic concentrations of acetylcholine *in vivo*, and it suggests that they are innervated by cholinergic axons. In addition, it indicates that the regulation of these [^{3}H]ACh recognition sites is similar to that of other neurotransmitter and hormone receptors that down-regulate in response to augmented stimulation.

Effects of chronic treatment with nicotinic agonists and antagonists. In contrast to cholinesterase inhibition, 10 days of nicotine treatment increased [^{3}H]ACh binding in cortex, thalamus, striatum, and hypothalamus (Table 5). Scatchard analyses indicated that the increase in binding was due to an increase in the number of [^{3}H]ACh recognition sites (Table 6). Muscarinic receptors labeled with [^{3}H]QNB were unaffected by nicotine treatment (Table 5).

The time course of the nicotine-induced increase in nicotinic cholinergic binding was examined in the cerebral cortex after either a single injection or two injections each day for

1-21 days (Fig. 3). There was no change in binding either 1 hr after a single injection or 18 hrs after 1 day of treatment. Following 5 days of treatment, however, [^{3}H]ACh binding was increased by 20 percent, and after 10 days of treatment the binding was increased by 28-30 percent. This increase was maintained following 21 days of treatment. The increased binding induced by nicotine treatment appears to be reversible. Thus, when rats were killed 7 days after a 10-day treatment period, the [^{3}H]ACh binding was still significantly increased compared to binding in brains from control rats, but it appeared to be returning to control values and was significantly lower than binding in brains from rats killed 18 hrs after the last treatment (Fig. 3).

To determine whether the increase in [^{3}H]ACh recognition sites after treatment with nicotine was due to activation of the receptor or simply to occupancy, rats were treated for 10-14 days with either the nicotinic agonist cytisine, the nicotine metabolite cotinine, or the antagonists mecamylamine or dihydro-ß-erythroidine. In each case, the effects of the treatment were compared to those of nicotine treatment. The treatment with cotinine did not effect binding (Table 7A), but treatment with cytisine increased binding to nearly the same extent as nicotine treatment (Table 7A). Neither of the antagonists altered binding significantly (Table 7, B-D). It was also important to examine whether the antagonists could prevent the increase in [^{3}H]ACh binding sites induced by nicotine. Therefore, rats were pretreated with mecamylamine or dihydro-ß-erythroidine 20 min before each injection of nicotine for 10 days. Neither antagonist prevented the increase in [^{3}H]ACh binding induced by nicotine (Table 7, B and C).

The mechanism of the nicotine-induced increase in [^{3}H]ACh nicotinic recognition sites in brain is of particular interest and importance. The effect appears to be selective because muscarinic receptors are not affected by nicotine treatment. In addition, the effect appears to be specific to nicotinic agonists because administration of cytisine, a ganglionic agonist, produces a similar effect, but administration of neither cotinine nor either of two antagonists altered the binding of [^{3}H]ACh. Further, pretreatment with the antagonists did not block the increase induced by nicotine. Thus, the data suggest that the critical site on the receptor from which the signal for up-regulation is triggered is distinct from the site where antagonists bind. This critical site could be the agonist recognition site itself.

Nicotine is classically defined as an agonist at cholinergic receptors in muscle and ganglia (9), and it appears to be an agonist in brain also (10,11). However, following exposure to nicotine there is a rapid decrease in cholinergic receptor responsivity in muscle and ganglia due to depolarization blockade (12,13). Following adequate doses of nicotine, diminished receptor responsivity persists beyond the depolarization phase (14), reflecting prolonged desensitization of the receptor. Therefore, it is possible that repeated administration of nicotine results in a protracted functional blockade of the receptor, and this could signal up-regulation by the cell.

3. Measurement of [^{3}H]ACh recognition sites in brain by autoradiography. We have used quantitative autoradiography to begin to map the distribution of [^{3}H]ACh recognition sites in brain. In this procedure, 32 um frozen sections of rat brain were cut with a cryostat, mounted onto slides, preincubated for two 10-min periods, and then incubated with [^{3}H]ACh under conditions similar to those that we use for homogenate binding. After incubation each

slide was washed twice for 5 min in cold buffer, rinsed in distilled water to remove buffer salts, dried on a slide warmer, and then juxtaposed to LKB Ultrofilm in x-ray cassettes. After exposure for 6-8 weeks, the films were developed and fixed in Kodak D-19. Optical densities of the brain regions on the films were measured with a microcomputer-assisted densitometer and compared with standards made from frozen brain homogenate to which known amounts of [^{3}H] were added.

The results of our initial autoradiographic studies confirm data from our homogenate binding studies. Thus, high binding was observed by autoradiography in the thalamus, and moderate levels of binding were observed in the cerebral cortex, striatum, and hippocampus. However, autoradiography has allowed quantitation of [^{3}H]ACh recognition sites in specific nuclei of the thalamus and specific layers of the cortex and the hippocampus. In addition, binding in very small areas of brain, in which studies of homogenate binding are not practical, is easily visualized and quantitated by autoradiography. For example, the medial habenula, which is too small for routine studies of homogenate binding, appears to contain the highest density of [^{3}H]ACh recognition sites in rat brain.

Autoradiography should contribute significantly to our knowledge of the distribution of the [^{3}H]ACh nicotinic recognition sites in brain, and to the effects of drugs such as nicotine and the cholinesterase inhibitors DFP and soman on those sites.

TABLE 1

Effects of disulfide bond and sulfhydryl reagents on [^{3}H]-acetylcholine binding in cerebral cortex

Cortical homogenates were incubated with 1 mM dithiothreitol, DTNB, or PCMB for 30 min at 0° (pH 8.5) and either washed for subsequent treatment with another reagent or prepared for [^{3}H]acetylcholine binding (10 nM) as described in the text. Control binding = 2.11 ± 0.08 pmoles/g of tissue. Data are expressed as the mean ± standard error of the mean of four experiments.

Pretreatment	% of control
Control	100 ± 3.8
A. 1 mM dithiothreitol	24.4 ± 8.0[a]
B. 1 mM DTNB	90.0 ± 8.0
C. 1 mM dithiothreitol followed by 1 mM DTNB	90.6 ± 4.1
D. 1 mM PCMB	107.6 ± 3.7
E. 1 mM dithiothreitol followed by 1 mM PCMB	33.7 ± 1.2[a]
F. 1 mM dithiothreitol followed by 1 mM PCMB followed by 1 mM DTNB	71.4 ± 5.7[b]

[a] $p < 0.01$ compared with control.

[b] $p < 0.01$ compared with control and with Treatments C and E.

TABLE 2

Effect of dithiothreitol pretreatment on agonist and antagonist competition for [^{3}H]acetylcholine binding sites in cerebral cortex

Cortical homogenates were incubated with 300 μM dithiothreitol for 30 min at 0° (pH 8.5). The reaction was terminated by dilution with cold buffer. The washed homogenates were washed and subsequently incubated with 10 nM [^{3}H]acetylcholine and several concentrations of competing drugs for 40 min at 0° as described in the text. The concentration of drug which decreased specific binding of [^{3}H]acetylcholine by 50% (IC_{50}) was determined graphically by inspection. Each value is the mean ± standard error of the mean of two to four experiments.

Competing drug	IC_{50}	
	Untreated	Dithiothreitol-treated
	nM	
Dihydro-β-eryhtroidine	215 ± 39	214 ± 31
d-Tubocurarine	38,000 ± 2,500	25,300 ± 5,800
Hexamethonium	630,000 ± 60,000	530,000 ± 30,000
Cytisine	5.0 ± 0.1	7.3 ± 0.3

TABLE 3

Effect of nicotinic cholinergic agonist pretreatment on the reduction of disulfide bonds by dithiothreitol in rat cerebral cortex

Cortical homogenates were incubated in the presence or absence of 100 μM acetylcholine (in the presence of 100 μM DFP) or 100 μM (−)-nicotine for 30 min at 0°. Subsequently, 1 mM dithiothreitol was added and the total mixture was incubated for an additional 30 min at 0° (pH 8.5). The reaction was terminated by dilution with cold buffer, and the homogenates were washed two times. [^{3}H]Acetylcholine binding (10 nM) was assayed in the washed homogenates as described in the text. Control binding = 1.81 ± 0.22 pmoles/g of tissue. The data are expressed as the mean ± standard error of the mean of three experiments.

Pretreatment	% of control
Control	100 ± 12.2
A. 1 mM dithiothreitol	40.7 ± 4.6[a]
B. 100 μM acetylcholine	99.3 ± 8.9
C. 100 μM (−)-nicotine	106.6 ± 5.6
D. 100 μM acetylcholine + 1 mM dithiothreitol	32.5 ± 2.1[a]
E. 100 μM (−)-nicotine + 1 mM dithiothreitol	43.0 ± 4.5[a]

[a] $p < 0.01$ compared with control.

TABLE 4 *Effect of repeated* DFP *administration (10 days) on [³H]ACh binding to nicotinic sites in several areas of rat brain*

Brain area	Specific binding (pmol/g tissue)	
	Control	DFP
Cerebral cortex	2.2 ± 0.06 (13)	1.7 ± 0.08[a] (11)
Thalamus	3.2 ± 0.17 (9)	2.6 ± 0.18[b] (10)
Striatum	2.2 ± 0.07 (6)	1.5 ± 0.05[a] (5)
Hypothalamus	0.72 ± 0.07 (4)	0.45 ± 0.06[b] (4)

Nicotinic sites were assayed using 10 nM [³H]ACh as described in the text. Cholinesterase activity was inhibited in the DFP-treated rats by ~82%. The data are expressed as means ± SEM for the number of animals indicated in parentheses. Data for cerebral cortex are from reference 15 and are presented here for purposes of compairson.

[a] $p < 0.01$ compared with control.

[b] $p < 0.05$ compared with control.

TABLE 5 *Effect of repeated nicotine administration (1 mg/kg twice daily for 10 days) on [³H]ACh binding to nicotinic sites and [³H]QNB to muscarinic sites in several areas of rat brain*

Brain area	Specific binding (pmol/g tissue)			
	[³H]ACh		[³H]QNB	
	Control	Nicotine	Control	Nicotine
Cerebral cortex	2.0 ± 0.04 (10)	2.5 ± 0.06[a] (10)	74.7 ± 1.5 (4)	74.5 ± 2.2 (4)
Thalamus	3.6 ± 0.11 (9)	4.6 ± 0.17[a] (8)	41.4 ± 1.4 (5)	39.5 ± 3.3 (5)
Striatum	2.1 ± 0.05 (16)	2.5 ± 0.24[a] (15)	86.3 ± 2.3 (5)	91.3 ± 3.0 (5)
Hypothalamus	0.67 ± 0.04 (7)	0.92 ± 0.07[a] (8)	—	—

Nicotinic sites were assayed using 10 n*M* [³H]ACh in each area. Muscarinic sites were assayed using 0.6 n*M* [³H]QNB in the cortex and thalamus and 0.2 n*M* in the striatum. The data are means ± SEM for the number of rats indicated in parentheses.

[a] $p < 0.01$ compared with control.

TABLE 6. *Effect of repeated nicotine administration on density (B_{max}) and affinity (K_D) of [^{3}H]ACh binding sites in three areas of rat brain*

Brain area	B_{max} (pmol g tissue)		K_D (n*M*)	
	Control	Nicotine	Control	Nicotine
Cerebral cortex	4.1 ± 0.31	5.3 ± 0.11[a]	8.3 ± 0.90	8.0 ± 0.47
Thalamus	6.9 ± 0.63	9.0 ± 0.31[a]	9.1 ± 1.0	10.9 ± 1.4
Striatum	4.6 ± 0.33	6.4 ± 0.40[a]	10.6 ± 2.0	11.1 ± 1.8

Brain homogenates were prepared from rats that were injected twice daily for 10 days with (−)-nicotine (1 mg/kg s.c.). The homogenates were incubated with [^{3}H]ACh (2.5–30.0 n*M*) at 0°C for 40 min as described in the text. The data are expressed as means ± SEM for four experiments.

[a] $p < 0.05$ compared with control.

TABLE 7 *Effects of repeated administration of nicotinic agonists and antagonists and a metabolite of nicotine on [^{3}H]ACh binding in the cerebral cortex*

Drug treatment	Specific binding (pmol/g tissue)
A	
Control	2.1 ± 0.05
Nicotine	2.9 ± 0.10[a]
Cotinine	2.1 ± 0.11
Cytisin	2.6 ± 0.09[a]
B	
Control	2.3 ± 0.07
Dihydro-β-erythroidine	2.2 ± 0.09
Nicotine	2.9 ± 0.06[a]
Dihydro-β-erythroidine + nicotine	3.0 ± 0.09[a]
C	
Control	1.3 ± 0.04
Mecamylamine	1.5 ± 0.12
Nicotine	1.6 ± 0.07[a]
Mecamylamine + nicotine	1.6 ± 0.06[a]
D	
Control	1.6 ± 0.10
Mecamylamine	1.7 ± 0.09

A: Rats were injected twice daily for 10 days with (−)-nicotine (1 mg/kg) or with equimolar doses (6 μmol/kg) of (−)-cotinine or (−)-cytisin. B and C: Rats were injected twice daily for 10 days with the antagonists dihydro-β-erythroidine (1 mg/kg) or mecamylamine (1 mg/kg), with nicotine (1 mg/kg), or with a combination of one of the antagonists followed 20 min later by nicotine. D: Rats were injected twice daily for 14 days with mecamylamine (3 mg/kg). The nicotinic binding sites were assayed using 7–10 n*M* [^{3}H]ACh. Data are the means ± SEM from six to eight rats. Tissues from each drug treatment group were assayed together in parallel with the indicated control group.

[a] $p < 0.05$ compared with control.

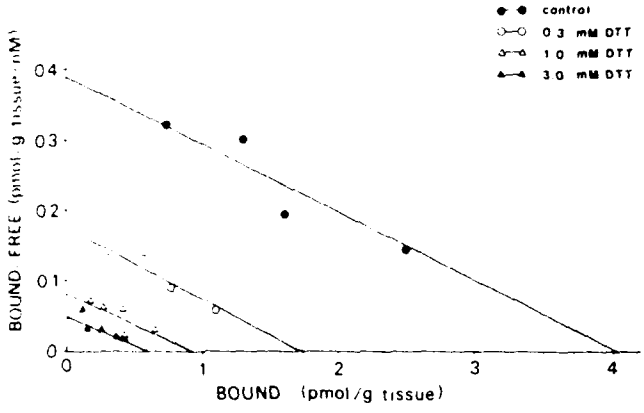

FIG. 1. *Scatchard analysis of [^{3}H]acetylcholine binding in dithiothreitol-treated cerebral cortex*

Cortical homogenates were incubated with 0.3, 1.0, or 3.0 mM dithiothreitol (*DTT*) for 30 min at 0° (pH 8.5). The reactions were terminated by dilution with cold buffer and centrifugation at 49,000 × *g* for 10 min. The treated homogenates were then prepared for the [^{3}H] acetylcholine binding assay as described in the text using 2.5–30.0 nM [^{3}H]acetylcholine. The scatchard plots are representative of 2–7 experiments. The K_D and B_{max} were determined by least-squares linear regression. The B_{max} value for the control was 3.7 ± 0.2 pmoles/g of tissue. Pretreatment with 0.3, 1.0, and 3.0 mM dithiothreitol decreased the B_{max} values to 1.8 ± 0.2, 1.3 ± 0.2, and 0.7 ± 0.1 pmoles/g of tissue, respectively. The K_D value for the control was 11.1 ± 1.3 nM. The K_D values following pretreatment with 0.3, 1.0, and 3.0 mM dithiothreitol were 11.4 ± 1.8, 12.1 ± 1.2, and 13.6 ± 2.6 nM, respectively.

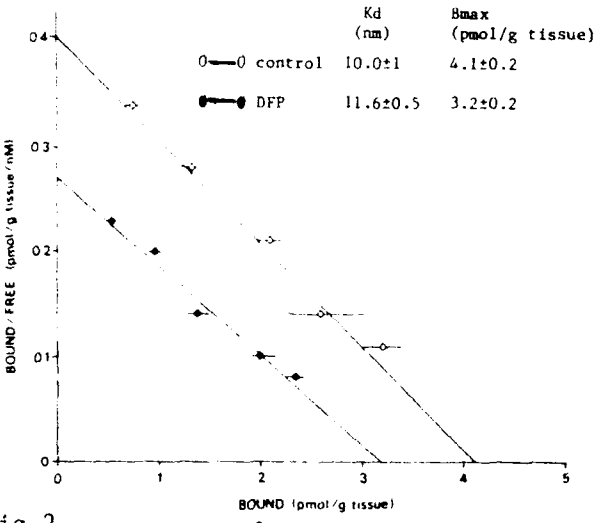

Fig. 2 Scatchard plots of [^{3}H]ACh binding in cerebral cortex from rats administered DFP for 10 days. Cortical homogenates were prepared from control rats and from rats that were injected with DFP as described in the text. The homogenates were incubated with varying concentrations of [^{3}H]ACh (2.5–30.0 n*M*). Each point represents the mean ± SEM of the abscissa and ordinate coordinates for four experiments. The B_{max} values of the DFP and control groups are significantly different ($p < 0.05$)

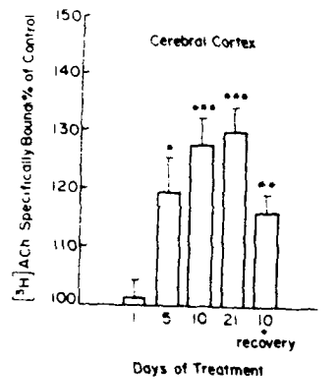

Fig. 3 Time course of the effects of repeated nicotine administration on [^{3}H]ACh binding in rat cerebral cortex. Cortical homogenates were prepared from rats that were injected twice daily with (−)-nicotine (1 mg/kg) for the number of days indicated. The rats were killed 18 h after the last injection except those in the "recovery" group, which were killed 7 days after the last injection. [^{3}H]ACh binding (10 n*M*) was assayed as described in the text and is plotted as a percentage of control binding. Data are expressed as means ± SEM for 6–14 rats. Control binding was 2.2 ± 0.1 pmol/g tissue. $^*p < 0.05$. $^{***}p < 0.01$ compared with control; $^{**}p < 0.05$ compared with the 10-day treatment group that was killed 18 h after the last injection.

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